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Adducted Proteins for Identification of Endogenous Electrophiles

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Chemically reactive compounds in tissues can be monitored through their products of reaction with biomacromolecules. For the purpose of *in vivo* dose monitoring, hemoglobin (Hb) has been preferred to DNA because of its well-defined life span and more facile chemical identification of adducts. Through the *N*-alkyl Edman method, adducts to the N-terminals (valines) of the globin chains are measured mass spectrometrically with high sensitivity. In studies of low molecular weight adducts from occupational exposures or tobacco smoke, background levels were found in nonexposed control persons. In some cases the origin of these adducts could be determined. For instance, the 2-hydroxyethyl adduct has been shown to originate from ethylene oxide, a metabolite of endogenously produced ethene. The measured level, about 20 pmole/g globin, agrees well with the ethylene oxide dose calculated from expired ethene. Animal studies indicate contributions from the intestinal flora and dietary factors. An average background level of about 200 pmole/g globin of methylvaline has been observed in unexposed humans. From reaction-kinetic studies of *S*-adenosylmethionine (SAM), it has been shown that the background mainly originates from SAM. In twin studies, a genetic influence on the level has been shown. Furthermore, a contribution from tobacco smoking to the level was demonstrated in these studies. Certain aldehydes, e.g., malonaldehyde, have been shown to be related to dietary factors and lipid peroxidation. These studies show the usefulness of the method in a search for reactive compounds in the body, with the ultimate goal of assessing the total genotoxic load.

Introduction

In vivo dose monitoring by means of adducts to macromolecules and characterization of electrophilic reagents of endogenous origin in humans not knowingly exposed to carcinogens is of interest for several reasons. Primarily, these studies are an important step in assessing the total load of mutagens and carcinogens in human populations and in developing preventive measures when the associated risks are high. Furthermore, studies of these kinds are required for the optimization of dose monitoring of low molecular weight exogenous carcinogens because background of adducts identical with the ones to be measured have been encountered in many cases (*1–3*).

With these purposes in mind, the present paper summarizes the present status of work on low molecular weight electrophiles in humans not knowingly exposed to carcinogens. With few exceptions, measurement of specific DNA adducts with sufficient sensitivity is not yet possible, and this is one reason why the work has focused on measuring adducts to hemoglobin (Hb). It should be remembered that demonstrated Hb adducts are a good surrogate for the corresponding DNA adducts (*4*), the relative rate of formation of which is easily established in animal experiments with labeled compounds.

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The *N*-alkyl Edman method (*1,5*) permits the measurement, with high resolving power, of specific adducts to the amino terminals, valines, of the globin chains. Of importance to the clarification of the origin of adducts to the N-terminals is that *N*-alkylvalines, as compared to *S*-alkylcysteines and N^{π}- or N^{τ} alkylhistidines, are not misincorporated in protein synthesis (*6*). Valine-*N* adducts therefore reflect *in vivo* formation through chemical reaction, provided that artifactual formation of the same adducts during storage and preparation of samples is controlled (*7*). Artifact formation, which has been demonstrated for adducts from simple epoxides and malonaldehyde, concerns other amino acids as well. Due to the mild derivatization conditions, the monitoring of valine adducts is advantageous because the formation of adducts [e.g., during acid or alkaline hydrolysis (*8*)] is avoided.

The *N*-alkyl Edman method involves isolation and derivatization of globin with pentafluorophenyl isothiocyanate, the resulting *N*-alkylamino acid pentafluorophenylthiohydantoin being measured by GC–MS. Details of procedures are given elsewhere (*1,5,9*). For *N*-alkylidenevalines (i.e., Schiff bases from aldehydes), derivatization is preceded by reduction to secondary amines (*10*).

N-Alkylvalines

N-(2-Hydroxyethyl)valine

Measurement. *N*-(2-Hydroxyethyl)valine (HOEtVal) has been extensively studied with the *N*-alkyl Edman method, ethylene oxide being used as a model carcinogen during method develop-

ment. Elevated levels of HOEtVal are observed as a result of occupational exposure to ethylene oxide or ethene, which is converted to ethylene oxide, and in smokers due to the content of ethene in the smoke (*11*). In all studies of exogenously exposed populations, a background level of HOEtVal has been measured in the unexposed control individuals. Similar background levels have been observed in laboratory animals. In early studies with Hb hydrolysates from occupationally unexposed persons, rather high background levels of hydroxyethylhistidines were observed (*12,13*). In studies of valine adducts with the more sensitive *N*-alkyl Edman method, it was shown that there was a large contribution (85 pmole/g globin per 10 cigarettes/day) to the background level of HOEtVal due to smoking (*9,14*). Still, the HOEtVal level in nonsmokers was rather high (25–110 pmole/g globin) (*14*), but with the development of the method the levels have decreased considerably. It appears that the lower, recent values around 20 pmole/g (*9*) are correct. The decrease from the earlier determinations is to some extent due to an improved resolving power in the analysis and mainly the avoidance of artifact formation of HOEtVal during storage.

Unexpectedly high background levels of HOEtVal found in a study of unexposed nonsmokers were shown to be due to adducts being formed as artifacts during storage of Hb samples (*7*). *In vitro* studies have shown that the formation of HOEtVal as an artifact is dependent on oxidative processes, may be with ethene as an intermediate (*7*). The process was shown to be enhanced by lyophilization of samples. Artifactual HOEtVal has been found at levels covering the whole range (up to 30 nmole/g globin) of what could be expected from occupational exposure to ethylene oxide. Therefore, precautions against artifact formation have to be taken in all studies of HOEtVal, particularly in studies of background levels. Storage as precipitated globins has so far been shown to be safe in this respect (*7*). The artifact problem is of importance to the extent that blood bank materials will be used for mapping background levels of adducts; this also concerns alkylidenevalines.

An intercalibration study between four laboratories measuring HOEtVal adducts illustrates the difficulties of attaining the resolving power needed for determining low background levels (*15*). The resolving power can be increased by technically improving the MS analysis, further purifying the derivatized samples (*16*), and/or enriching adduct-carrying chains (*17*).

In 10 studies involving a total of about 110 persons, where artifact formation and contamination were carefully controlled and occupationally exposed individuals and smokers were excluded, a mean background level of 18 pmole/g globin, range 8–30 pmole/g, was obtained. There are strong reasons to assume that these values are correct. A few analyses on mice and rats fed standard laboratory diets give about the same values. Background levels in the same individual may vary, as shown by measurements over a 15-month period (Table 1). It is possible ($p \approx 0.04$) that sample no. 4 was preceded by an exposure or that it reflects an increased endogenous production of ethene/ethylene oxide.

The contributions to uncertainty were measured in a couple of studies showing the coefficients of variation (CV) for derivatization, injection, and reading to be 4.7, 7.8, and 4.5%, respectively, corresponding to a total CV of 10.2%. Greater variation may occur on occasions with unfavorable analytical conditions, which, however, can be detected. A systematic error (due to uncertainty in the true level in the standard proteins) may amount to 15%.

Table 1. Variation during 15 months in the background level of HOEtVal in blood samples collected on different occasions from one person.

	Sample number					
	1	2	3	4	5	6
HOEtVal, pmole/g[a]	18.6	22.7	21.4	28.2	24.6	18.9

HOEtVal, *N*-(2-hydroxyethyl)valine.
[a]Mean, 22.7 pmole/g; coefficient of variation, 14.7%.

Origin. It has been shown earlier that ethene is endogenously formed in living organisms including man (*18*). Experiments were conducted to determine the extent that endogenously formed ethene could give rise to the observed background of HOEtVal in humans (*19*). The ethene content in the exhaled air and the HOEtVal content in Hb were determined in unexposed nonsmokers, and in the same individuals the inhalation pharmacokinetics of ethene were studied in an independent experiment. Pharmacokinetic parameters for ethylene oxide were known from previous studies. By the application of a pharmacokinetic model to these data, the steady-state level of ethylene oxide was calculated to be 0.17 nmole/L tissue. The exposure to environmental ethene, estimated to 15 ppb, would give a further contribution of 0.08 nmole/L tissue, the total dose of ethylene oxide, $[EO]_{tot}$, being 0.25 nmole/L tissue.

The valine-adduct level, [R-Val], is determined by the rate constant, k_{val}, mean concentration, $\bar{C}$, and time, t, according to

$$[\text{R-Val}] = k_{val} \cdot \bar{C} \cdot t \qquad (1)$$

where $\bar{C} \cdot t$ = dose (D).

In chronic or intermittent exposure, a steady-state adduct level, $[\text{R-Val}]_{ss}$, is reached in one erythrocyte life span, t_{er} = 18 weeks, the steady-state adduct level corresponding to the cumulative level during the time $t_{er}/2$. From k_{val} for ethylene oxide [5×10^{-5} L/g · hr (*20*)], the expected level of HOEtVal may thus be calculated according to

$$[\text{HOEtVal}]_{ss}(\text{mole/g}) = k_{val} \cdot [EO]_{tot} \cdot \frac{t_{er}}{2} = 5 \cdot 10^{-5}(\text{L/g·hr}) \cdot 0.25 \cdot 10^{-9}(\text{mole/L}) \cdot \frac{18 \cdot 168}{2}(\text{hr})$$

$$\approx 19 \cdot 10^{-12}\ \text{mole/g}$$

$$= 19\ \text{pmole/g}$$

This is in agreement with the value of HOEtVal in the studied persons, 20 ± 4 (SD) pmole/g globin.

A few exploratory experiments with mice were carried out to shed light on determinants of the background level of HOEtVal (*21*). A lower value of HOEtVal (by about 10 pmole/g) was obtained in germ-free mice as compared to control animals. In another experiment, a higher level (by about 15 pmole/g) was observed in mice fed an unsaturated-fat diet (sunflower seed oil) compared to mice fed a saturated-fat diet (coconut oil). In more recent studies of different fat diets and induction of lipid peroxidation, only a slightly significant increase (about 1 pmole/g) was found in mice fed the unsaturated-fat diet (soya oil) (*22*). However, mice fed a normal laboratory diet have shown a higher

background (a few picomoles) than mice fed a soya-oil diet (unpublished data), which indicates that the soya-oil diet is unfavorable in studies with this purpose. These findings, together with the human studies, are compatible with endogenously produced ethene being the major source of background HOEtVal. Ethene has been shown to be produced by enteric bacteria and in peroxidation of lipids and methionine (*7,18,21*).

N-(2-Hydroxypropyl)valine

Hydroxypropyl adducts to histidine and valine have been measured in propylene oxide-exposed persons (*23*). An increased level of *N*-(2-hydroxypropyl)valine (HOPrVal) has been determined in animals with exposure to propene in motor exhausts (*5*). In smokers, an increment of HOPrVal of about 2 pmole/g globin per 10 cigarettes/day has been observed (*24*). Nonsmokers show a background of about 2 pmole/g of HOPrVal (*24*). However, there is still an uncertainty by at least a factor of 2 in the absolute quantification of HOPrVal. The difficulties of measuring low levels of HOPrVal depend above all on the resolving power attained with the standard analytical procedure being insufficient. HOPrVal has so far not been shown to be formed as an artifact during storage, but has been shown to be formed during globin precipitation from an acetone-HCl solution in the range of 10 pmole/g, probably from the acetone reacting in the enol form. It is unlikely that propene in environmental tobacco smoke (*25*) and urban air pollution (*26*) account for the observed background levels of HOPrVal. Pathways for endogenous production of propene or some other precursor are at present not known.

N-Methylvaline

A relatively high background level of *N*-methylvaline (MeVal) in Hb, about 500 pmole/g, was observed in an earlier study (*27*). Due to interindividual variation of this background, the expected increment from methylators in tobacco smoke was not detectable with statistical significance in smokers. This situation was improved in a later study (*28*) in which twin pairs discordant for smoking were investigated. The study showed an increment from smoking of about 3.5 pmole/g per cigarette/day to be highly significant ($p < 0.001$) when it was calculated as the within-pair difference between smoking and nonsmoking twins. In addition, the hypothesis that the variations in the background were partly hereditary was confirmed ($p < 0.001$; Fig. 1). It is noteworthy that a correlation between the background levels of MeVal and HOEtVal (*28*) ($p < 0.05$) and perhaps also HOPrVal is indicated. This might be interpreted by the existence of a common link, such as detoxification, in the metabolism of the different alkylators.

In this later study (*28*), the background level of MeVal was found to be lower (mean 220, range 150–300 pmole/g). This was partly due to improved calibration, a step which has been somewhat cumbersome in the analysis of MeVal (*9,27*). MeVal has not been shown to be formed as an artifact during storage. The observed high levels of *S*-methylcysteine (*29*) and ring-*N*-methylhistidines (*27*) might be due to misincorporation (*6*).

In the previous study (*9,27*), *S*-adenosylmethionine (SAM) was indicated to be a possible source of background methylations. A determination of the second-order rate constant, k_{val},

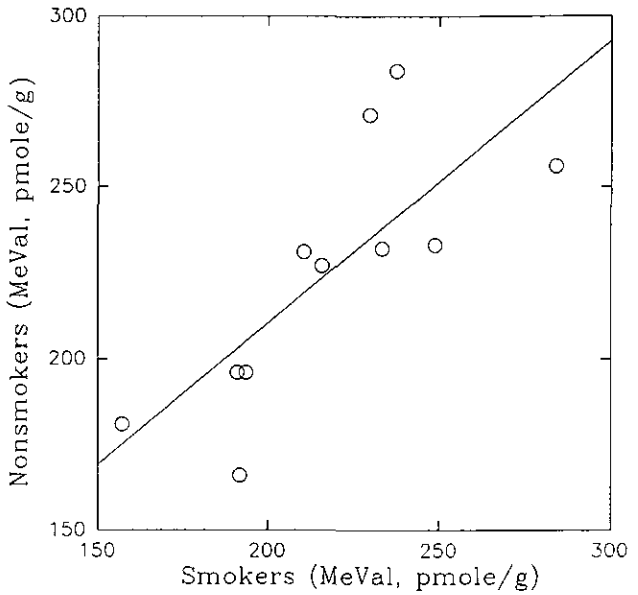

FIGURE 1. Correlation of the background levels of *N*-methylvaline (MeVal) in monozygotic twins discordant for smoking. Levels in smoking twins adjusted for the contribution from smoking [estimated to be 3.5 pmole/g per cigarette per day; Törnquist et al. (*28*)].

for methylation of the N-terminal of Hb confirmed this hypothesis (manuscript in preparation). From the value of the constant, $k_{val} = 3 \times 10^{-8}$ (L/g · hr) and the concentration of SAM in erythrocytes, 3.5 μM (*30*), the expected level of MeVal is calculated according to Equation 1 to be 160 pmole/g.

This expectation is compatible with the observed levels. Another cellular methylator, choline, was found to react at least 10^4 times more slowly than SAM. Even if the concentration in erythrocytes of choline is higher than that of SAM by one to two orders of magnitude, this source is expected to give a small contribution only. This is also the case with methyl chloride, partly formed from SAM (*27*).

N-Alkylidenevalines

Reactivity of Aldehydes

Saturated aldehydes react reversibly with amino groups (e.g., N-terminal valine in Hb) through Schiff-base formation. α,β-unsaturated aldehydes can also react through 1,4-addition. Schiff-bases can be transformed to stable secondary amines by reduction with $NaBH_4$, for example. α-Hydroxycarbonyl compounds, such as glycolaldehyde, give rise to stable 2-oxoalkylamines through Amadori rearrangement of initially formed Schiff-bases (*31*), and in the reduction of these amines 2-hydroxyalkylamines are formed. Schiff-bases may also rearrange to stable cyclic imidazolidinone derivatives that are not reducible (*32*).

Measurement of Adducts

Hemoglobin adducts from aldehydes can be monitored by the *N*-alkyl Edman method after reduction with $NaBH_4$ to secondary amines (see Table 2). Considering that aldehyde adducts are

Table 2. Adducts to N-terminal valine in human hemoglobin from some endogenously formed aldehydes.

Reduced adduct to N-terminal valine	Formula	Mean (range), nmole/g	Unreduced adduct	Origin
Methyl	CH_3-	~ 4 (2–12)	$CH_2=$	Formaldehyde
Ethyl	CH_3CH_2-	~ 2 (1–5)	$CH_3CH=$	Acetaldehyde
2-Hydroxyethyl	$HOCH_2CH_2-$	~ 3 (2.5–5)	$HOCH_2CH=$	Glycolaldehyde
2-Hydroxypropyl	$CH_3\underset{\underset{OH}{|}}{C}HCH_2-$	~ 7 (4–10)	$CH_3\overset{\overset{O}{\|\|}}{C}CH=$	Methylglyoxal
3-Hydroxypropyl	$HOCH_2(CH_2)_2-$	~ 0.3 (0.2–0.9)	$CHCH=CH-\underset{\underset{O}{|}}{}$	Malonaldehyde
Hexyl[a]	$CH_3(CH_2)_5-$	~ 0.01 (0.0–0.02)	$CH_3(CH_2)_4CH=$	Hexanal

[a]Determined as described by Kautiainen (*35*).

unstable and that artifact formation could present problems in analysis, work-up procedures for blood samples to be analyzed for aldehyde adducts were carefully studied. It was found that samples had to be reduced as hemolysate to avoid contamination from aldehydes present in solvents and to avoid possible loss of reversibly bound aldehyde adducts (*10*). The reproducibility of the analysis has been studied with regard to the added amount of $NaBH_4$, time of storage, etc. Furthermore, the variations of one person's adduct levels were studied. It was concluded that the measurement of methyl and ethyl adducts was less reproducible than the adducts from other aldehydes. So far, the reason for the large fluctuations in the levels of methyl and ethyl adducts is not known. The formation of precursors of these adducts (i.e., formaldehyde and acetaldehyde) during the reduction of hemolysate cannot be excluded.

Malonaldehyde (MA) has been shown to be formed in stored blood (*33*) and in blood plasma (*34*). High levels of MA adducts (up to 40 nmole/g globin) were measured in samples from red blood cells stored frozen for several years. Also high levels of the adduct from hexanal, another aldehyde formed by lipid peroxidation, were found in these samples. These results illustrate that, because peroxidation processes may proceed in frozen biological material, blood samples should not be stored as frozen red blood cells.

Mean values and ranges of levels of adducts obtained in unexposed persons from some aldehydes that are formed endogenously as intermediates in biochemical reactions are listed in Table 2. Current studies indicate that the levels of methyl and ethyl adducts can be influenced by different treatments of animals. This indicates that, in spite of the analytical difficulties described, the adduct levels measured are approximately correct.

Origin

Major sources of endogenous formaldehyde are metabolism of glycine and serine. Other compounds that have been found to yield formaldehyde include choline, dimethylaminoethanol, dimethylglycine, and methionine (*36*). Acetaldehyde is the first metabolite of ethanol, and raised Hb adduct levels have been seen in alcoholics (*37*). Acetaldehyde is produced endogenously by intestinal bacteria from carbohydrates (*38*). Glycolaldehyde is formed as an intermediate in the oxidative degradation of the amino acids tryptophan, tyrosine, and phenylalanine (*39*). It is also formed when ethanolamine is deaminated to glycine (*40*). The pathway by which methylglyoxal is formed is less known, but it can be formed in mammalian tissues, via the aminoacetone cycle, from glycine and threonine. In some tissues methylglyoxal formed enzymatically from acetoacetate, lactaldehyde, and aminopropanol (*41*). Malonaldehyde and hexanal are produced by peroxidation of unsaturated fatty acids, especially arachidonic acid (*42*). Malonaldehyde is also formed in the biosynthesis of prostaglandins (*43*).

The adducts of MA have been the most extensively studied. The background level of MA adduct to valine (MA–Val) in human Hb is about 0.3 nmole/g globin, and in mice and rats fed standard diet it is about 4 and 0.6 nmole/g globin, respectively. Increased levels of MA–Val have been observed in mice after the induction of lipid peroxidation with carbon tetrachloride (*10*). The influence of the dietary fatty-acid composition showed that mice fed a soya-oil-based diet (rich in polyunsaturated fatty acids) had a higher background level of MA–Val than mice fed a coconut-oil-based diet (rich in saturated fatty acids) (*22*). The levels of adducts were 4.5 nmole/g and 2.9 nmole/g globin in animals fed a soya-oil- and a coconut-oil-based diet, respectively. Mice fed the soya-oil-based diet also showed increased susceptibility to induced lipid peroxidation. In a current study, no difference in the MA–Val level has so far been seen between smokers and nonsmokers (Törnqvist et al., unpublished data).

Assuming that the adducts of MA to N-terminal valine are stable and using the second-order rate constant for the reaction of MA with valine, k_{val} [3.2×10^{-4} L/g·hr (*10*)], the adduct level can be used for the calculation of the average concentration (Eq. 1). The values obtained for the concentration of free MA in red blood cells was 30 nM in mice (t_{er} = 40 days) fed standard laboratory diet and 0.7 nM in humans. The difference between the levels in mice and humans might reflect the metabolic rates of the different species, which, as shown by Adelman et al. (*44*), correlates with oxidative damage. A few other aldehydes, in addition to MA and hexanal, which are formed peroxidatively from

Table 3. Adducts from endogenous alkylating agents to N-terminal valine in hemoglobin from unexposed persons.

Adduct to N-terminal valine	Mean (range), pmole/g	Origin
2-Hydroxyethyl	18 (8–30)	Endogenously produced ethene
2-Hydroxypropyl	~ 2 (<1–~10)	?
Methyl	220 (150–300)	*S*-adenosylmethionine

arachidonic acid, have been determined by the *N*-alkyl Edman method *in vitro* (*35*).

Discussion

Background adducts (exemplified in Tables 2 and 3) may reflect a carcinogenic load. This appears from an assessment of the contributions to the cancer risk of the doses associated with observed adduct levels. The cancer risk associated with a HOEt-Val background level of 20 pmole/g globin may be estimated as follows. This steady-state level corresponds to the accumulated dose during one-half of the erythrocyte life span and is inversely proportional to the rate constant, k_{val}, for adduct formation. The annual dose, D_{ann}, expressed in millimolar-hours per year (mMhr/year) will hence be

$$D_{ann} = \frac{52\ (\text{weeks/year}) \cdot 20 \cdot 10^{-12}\ (\text{mole/g}) \cdot 10^{3}\ (\text{mM/M})}{9\ (\text{weeks}) \cdot 5 \cdot 10^{-5}\ (\text{L/g} \cdot \text{hr})}$$

$$= 2.3 \cdot 10^{-3}\ \text{mM-hr/year}$$

This dose may be assumed to be homogeneously distributed in the body (*45*). One approach to estimate the cancer risk associated with an observed adduct level is by expressing the chemical dose in radiation dose equivalents, RadEq (*46*). For ethylene oxide this value has been determined to 80 RadEq/mMhr (*47*). The calculated dose equivalent would thus amount to $0.0023 \cdot 80 = 0.18$ RadEq/year.

The National Research Council (*48*) estimated in its BEIR V report, from the multiplicative model applied, that in a population of the current age distribution in the United States irradiation with 1 msv (=0.1 rad low-LET radiation) per year would increase the number of cancer deaths by about 3%, i.e., by about 600 cases per million population. This model is also applicable to genotoxic chemicals (*49*), and an ethylene oxide dose of 0.18 RadEq/year would accordingly increase the risk by about 5%. However, in its calculation of risk for solid tumours, which make up the major part of the total cancer death rate, the BEIR committee did not allow for the lowering of effectiveness at lower dose rates. This factor is certainly at least 3, most probably around 5 (Ehrenberg et al., in preparation; *50*) and it is therefore prudent to give the risk associated with the HOEtVal background as being on the order of 1% of the current cancer mortality (*9*). For 2-hydroxypropylvaline the level is lower than that of HOEt-Val by one order of magnitude, and the associated risk, assuming the proximal reactive intermediate to be propylene oxide, may be assumed to be proportionally smaller.

The identification of SAM as the main causative agent for MeVal is here taken to exemplify the usefulness of adduct quantification and application of reaction kinetics in the clarification of origin of background adducts. For the next step, risk estimation, essential data are still lacking, although reaction with DNA *in vitro* has been demonstrated (*51*). The positively charged sulfonium ion cannot be assumed to be homogeneously distributed in the same way as a compound such as ethylene oxide. The distribution in the body of a natural methylator such as SAM is probably compartmentalized, with a protection of DNA against unwanted methylations. For this reason and because of the existence of an effective system (methyl transferase) repairing such methylations, conclusions about cancer risks due to chemical methylations by SAM would require special studies (*9*).

Due to their reactivity with DNA bases and their cross-linking action and the mutagenic activity demonstrated for many aldehydes (*52,53*), there are indications that these compounds may play a role in cancer initiation (*54*). The estimation of cancer risk from the doses of endogenously formed aldehydes, as measured by Hb adducts, cannot be done currently. Studies of the relationships between tissue dose (adduct level) and target dose and of the relative genotoxic potency of the respective aldehydes are fundamental to such estimation. It cannot be excluded that endogenously formed aldehydes give a considerable contribution to the total load of genotoxic agents.

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